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immunological characterizations of these three toxin components.

PRODUCTION OF HIGH LEVELS OF <u>BACILLUS</u> <u>ANTHRACIS</u> TOXIN ANTIGENS

IN A NEW, DEFINED CULTURE MEDIUM

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Short title: Defined Culture Medium for <u>B. Anthracis</u>

In conducting the research described in this report, the investigators

adhered to the "Guide for the Care and Use of Laboratory Animals," as

promulgated by the Committee on Care and Use of Laboratory Animals of

the Institute of Laboratory Animal Resources, National Research Council.

The facilities are fully accredited by the American Association for

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ABSTRACT

Improved cultural conditions and a new, completely synthetic medium (R medium) were developed to facilitate the production of Bacillus anthracis holotoxin antigens. Levels of these antigens, up to five-fold greater than the highest previously reported values, have been produced using the described system. The R medium was shown to be superior to 1095 and casamino acids media for the elaboration of lethal factor and protective antigen. The Sterne, V770-NP1-R, and Vollum 1B strains of B. anthracis were cultured for 42 h in R medium. Growth, pH change, glucose utilization, and supernatant protein concentration, lethal toxin activity and protease activity were monitored during this period. Lethal toxin production in all three strains was first detectable in late log phase and reached its maximum level during the stationary phase. Proteolytic activity, presumably responsible for degradation of the toxin, was detected in the supernatants of all three strains. The use of R medium to produce larger quantities of B. anthracis edema factor, protective antigen, and lethal factor will facilitate the biochemical, biophysical and immunological characterizations of these three toxin components.

The tripartite toxin of Bacillus anthracis is a complex toxin or toxic mixture (4) composed of three polypeptide factors (2, 23): edema factor (EF; factor I), protective antigen (PA; factor II) and lethal factor (LF; factor III). None of the factors alone possess demonstrable toxic activity (6, 14, 21), however intravenous injection of PA plus LF kills mice (21), rats (1, 2), and guinea pigs (22), while intradermal injection of PA plus EF produces edematous lesions in the skin of guinea pigs (8) and rabbits (24). LF, PA, and possibly EF are individually immunogenic. Parenteral administration of combinations of the three factors, or of LF or PA elicits varying degrees of protection from toxin or spore challenge in experimental animal hosts (15, 23).

In order to study the mechanisms of action of anthrax toxin and its role in B. anthracis pathogenesis, and as a prerequisite to vaccine development, investigators have devoted considerable attention to the production and purification of the three toxin components. Gladstone's demonstration in 1948 that B. anthracis produced an immunizing antigen when cultured in vitro under carefully controlled conditions (9) stimulated other researchers (3, 16, 30) to delineate specific cultural conditions affecting elaboration of the antigen. It was not until 1958, however, that Harris-Smith et al. (11) first demonstrated in vitro formation of an anthrax holotoxin possessing both lethality-producing and edema-producing activities. Subsequent attempts to improve both growth media and cultural conditions for production of protective antigen (17, 18, 31) or whole toxin (26, 27) led to the development of the 1095 medium of Wright et al. (18) and the casamino acids medium described by Haines et al. (10). Unfortunately, rather low yields of protective antigen are produced in 1095 medium (12), while the casamino acids medium is incompletely defined and contains activated charcoal powder (10).

The studies reported here accomplished three primary goals: (i) improved cultural conditions and a completely defined growth medium for B. anthracis in which EF, PA, and LF are elaborated at high levels; (ii) a comparison of production of lethal toxin by strains of B. anthracis cultured in 1095 and casamino acids media, and the new, defined medium; and (iii) examination of the parameters of B. anthracis growth, toxin formation, carbohydrate utilization, and protease production over a 42-h period of culture in the new medium. The results presented here demonstrate that with the new, defined medium, anthrax toxin antigens can be produced in vitro at levels greater than previously reported.

MATERIALS AND METHODS

Bacterial strains. A virulent, encapsulated strain of Bacillus anthracis (Vollum 1B) and two hypovirulent, nonencapsulated strains (Sterne and V770-NP1-R), were obtained in lyophilized form from the culture bank of the United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland. Vegetative organisms were stored frozen until use at -70°C in brain heart infusion (Difco Laboratories, Detroit, Mich.) plus 12% glycerol.

Media. Trypticase soy agar (TSA) was obtained from Baltimore Biological Laboratories (BBL, Cockeysville, Md.). Plates of sheep blood agar (SBA) were prepared using blood agar base (Difco) plus 5% sterile, defibrinated sheep blood. Casamino acids (CA, 27), 1095 (18), and synthetic R media were employed in these studies. Their composition is listed in Table 1. The components of all media were chemically pure or reagent grade and were obtained from Sigma Chemical Company (St. Louis, Mo.). The R medium was developed by modifying the CA medium in the following ways: (i) the quantities of individual amino acids found in 3.6 g of yeast extract were substituted for 3.6 g of casamino acids (19); (ii) the concentration of glucose was raised from 0.2 to 0.25%; (iii) monobasic potassium phosphate was omitted, and the concentration of dibasic potassium phosphate was increased from 0.088 to 0.3% to provide increased buffering; and (iv) activated charcoal powder was omitted.

Each liter of R medium contained the following components: (i) L-tyrosine, potassium phosphate, sodium-L-glutamate, and L-aspartic acid dissolved in 450 ml triple-distilled water heated to 60°C; (ii) 1 ml of a 1000-fold concentrated stock solution of cystine in 1.0 N HCl; (iii) 25 ml of 40-fold concentrated stock solutions of each of the

remaining components except sodium bicarbonate; and (iv) triple-distilled water to bring the solution volume to 995 ml. Sodium bicarbonate was dissolved into the solution and pH was adjusted to 8.0 with 5.0 N NaOH. The medium was filter-sterilized under positive pressure using a capsule filter with a 0.45 μ pore diameter (Gelman Sciences, Ann Arbor, Mich.) and stored at 4°C in tightly capped Erlenmeyer flasks. Medium prepared and stored in this manner was stable for at least 2 weeks.

Production of high level of toxin antigens. Environmental conditions favoring production of toxin were empirically developed during formulation of the R medium. Blood agar plates were inoculated with B. anthracis and incubated for 12 to 16 h at 37°C in 5% CO_2. Individual colonies were suspended in 10 ml sterile phosphate buffered saline (PBS) to a density of approximately 10^6 colony-forming units (CFU)/ml (approximately 0.01 absorbance units at 540 nm on a Coleman Junior spectrophotometer, using a 1.0-cm light path). Erlenmeyer flasks, half-filled with R medium, were inoculated with 1.0 ml of the B. anthracis suspension per 500 ml of medium, yielding an initial concentration of approximately 2×10^3 CFU/ml. Flasks were tightly capped to prevent loss of CO_2, then incubated in a controlled environment shaker (New Brunswick Scientific, New Brunswick, N.J.) at 37°C with 60 oscillations/min. Following 16 to 20 h incubation the cultures were centrifuged (500 X g, 15 min), and the supernatants filtered through cellulose acetate membranes (0.45 μ pore diameter, Gelman). These crude toxin preparations were stored up to 6 h on ice, or for longer periods at -70°C (8). On some occasions 2-liter Erlenmeyer filter flasks were used for culturing large quantities. The flasks were tightly sealed with rubber stoppers, and the side arms were plugged with vaccine bottle stoppers. At predetermined times flasks were removed, gently swirled and samples taken via the side arm using a 20-cc syringe

and a 21-gauge needle. The flasks were then reincubated for further sampling at the specified times.

Serological assay. Serological activity of crude toxin preparations was measured in an Ouchterlony double-diffusion system (26) using antiserum obtained from goats injected with B. anthracis Sterne spores. The goat antiserum possessed both anti-PA and anti-LF activity (S. Leppla, personal communication). Protective antigen and LF titers were expressed as reciprocals of the maximum dilution of culture supernatant yielding a precipitin line against goat antiserum diluted 1:2 with PBS.

Biological assays. Lethal toxin activity was measured by injecting culture supernatant into the dorsal penile vein of 200 to 300-g Fischer 344 rats and recording time to death (2). The potency of crude toxin preparations was expressed as toxic units (TU)/ml and was determined according to the method of Haines et al. (10).

Edema-producing activity was measured by intradermal injection of serial 2-fold dilutions of culture supernatants into the shaved backs of 300 to 400-g Hartley strain guinea pigs and noting edema formation 16 to 20 h later (27). Edema-producing activity titers were expressed as the reciprocals of the final dilution yielding a positive response.

Determination of growth and culture viability. Serial ten-fold dilutions of culture samples were spread in triplicate onto plates of TSA, 0.1 ml per plate. After 18 h incubation at 37°C, colonies on the plates were counted and CFU/ml in the original culture samples were determined.

Glucose and protein assays. Total glucose in the culture supernatants was determined by the method of Dubois et al. (5). Total protein in the culture supernatants was determined by the method of Sedmak and Grossberg (20).

Protease assay. Proteolytic activity in B. anthracis culture supernatants was assayed at pH 7.5 using a heterogeneous mixture of uniformly labeled [^{14}C] E. coli proteins as substrate (25, New England Nuclear, Boston, Mass.). This system is capable of detecting both endopeptidase and exopeptidase activity, and its sensitivity and ability to be used within a wide pH range make it suitable for quantitating low-level proteolytic activity in bacterial culture supernatants. As a positive control, trypsin (3 X crystallized, Sigma) was assayed in the system at concentrations of 0.1 to 0.0001 ug/ml.

RESULTS

Kinetics of growth in the R medium. Growth characteristics of the three B. anthracis strains in the new, defined medium are depicted in Fig. 1. All three strains reached stationary phase, approximately 2×10^8 CFU/ml, after 16 to 20 h of culture. Logarithmic phase doubling time was 35 to 40 min for the Sterne and V770-NP1-R strains, and approximately 50 min for the Vollum 1B strain. The viability of Sterne and V770-NP1-R decreased to about 10^7 CFU/ml between 20 and 42 h. Microscopic examination of material from the Vollum 1B culture revealed marked conversion of long chains into shorter chains and individual bacilli beginning 24 h postinoculation. This resulted in a temporary increase in viable count, followed by a decrease to 4×10^7 CFU/ml at 42 h.

Glucose utilization and pH change during culture. The rate of glucose utilization was similar in the three B. anthracis strains (Fig. 2). By 16 h more than 95% of the glucose had been depleted from the medium. Concomitant with the decrease in glucose was a drop in pH in the cultures to between 7.2 and 7.4 (Fig. 3). Measurements of pH were hampered by the tendency of the medium to rapidly lose CO_2 upon exposure to air, resulting in an increased pH.

Supernatant protein and proteolytic activity. Concentrations of supernatant protein in the three cultures increased slowly up to 24 h (Fig. 4). In stationary phase the protein concentrations for V770-NP1-R, Vollum 1B, and Sterne, were 34, 60, and 75 ug/ml. In the late stages of growth extensive cell lysis correlated with an increase in the supernatant protein concentrations. Low levels of proteolytic activity were demonstrable in the culture supernatants of all three strains (Table 2).

Elaboration of lethality-producing and edema-producing activities in culture. Lethality-producing activity in the culture supernatants of the three strains is shown in Fig. 5. Toxin was first detectable at 12 h, when cultures were in late log phase. Sterne, V770-NP1-R, and Vollum 1B demonstrated peak toxin activities of 70, 75, and 160 TU/ml at 16, 22, and 20 h respectively. Peak lethality-producing activity coincided with peak edema-producing activity titers of 16 for Vollum 1B and 8 for Sterne and V770-NP1-R.

Production of B. anthracis toxin antigens in 1095, casamino acids, and R media. In the preceeding experiment, in which numerous parameters of B. anthracis in R medium culture were examined, it was necessary to mix the contents of the 2-liter culture flasks immediately before removing samples for analysis. In subsequent studies, however, it was determined that more stable and higher titers of toxin biological activity were obtained when the cultures did not receive such intermittent mixing, but instead were incubated with gentle agitation and thoroughly mixed only once, directly before harvest of the supernatant at 16 to 20 h of culture.

In a final set of experiments, only one sample was taken from any single culture flask. Erlenmeyer flasks (250 ml) were half-filled with R, 1095, or CA medium, inoculated with B. anthracis Sterne, V770-NP1-R or Vollum 1B, and incubated with gentle agitation. Culture supernatant samples from individual flasks were harvested after 14, 16, 18, 20, 22 and 24 h of incubation and examined for lethality-producing activity and LF and PA. Maximum serologic and biologic activity levels attained are listed in Table 3. The highest PA and LF serologic activity titers consistently correlated with maximum lethal toxin activity levels

produced by the three strains cultured in the three media. In the 1095

medium, all three strains produced little serologically detectable PA,

no detectable LF, and very little lethal toxin. In the CA medium,

Sterne and Vollum 1B produced substantially more LF, PA, and lethal

toxin than in the 1095 medium, while there was no appreciable change

in toxin antigen production by V770-NP1-R. For all three strains the

highest levels of PA, LF, and lethal toxin were produced in the R medium.

DISCUSSION

Previous efforts by investigators to produce lethal anthrax toxin

in vitro have resulted in culture supernatant preparations containing

fewer than 40 TU/ml (1, 10, 13, 28, 29). By use of improved cultural

conditions and the R medium, we are now able to produce the toxin at

levels which are 2-fold (Sterne and V770-NP1-R strains) to 5-fold

(Vollum 1B) greater than the highest values previously reported. In

addition to its ability to support elaboration of high quantities of

anthrax toxin antigens, an additional advantage of the R medium is that

its nutritional components are completely defined.

Johnson and Spero reported that B. anthracis V770-NP1-R produced

only protective antigen in 1095 medium (12). We have demonstrated in these

studies that the V770-NP1-R strain produces very little PA or LF in either

1095 or CA medium. In the R medium, however, V770-NP1-R produces almost as

much lethal toxin as the Sterne strain. It is therefore apparent that the

specific cultural environment must be taken into consideration before

comparing relative toxin antigen production by strains of B. anthracis.

In the kinetic studies, attainment of stationary phase in the cultures

of the three strains correlated with depletion of supernatant glucose,

cassation in the rapid decline in supernatant pH, temporary plateau in
the increase in supernatant protein concentrations, and peak concentrations
of toxin factors in the supernatants. Lethal toxin activity in the three
strains was not detected until late log phase, approximately 5×10^7 CFU/ml.
Although this confirms previous findings (12), it may well be that toxin
antigens are elaborated throughout log phase culture but are not detected
due to the limited sensitivity of the biological assays employed (11, 29).
To obviate this problem, the development of more sensitive serologic and
biologic assays is currently being undertaken.

Degradation of crude and partially purified preparations of anthrax
toxin, presumably by proteases, has been reported by several investigators
(7, 8, 11, 29). It was therefore not surprising that proteolytic
activity was demonstrable in the culture supernatants of all three
strains. We have also noted (unpublished observations) that when the pH
of B. anthracis cultures falls below 7.0, either as a result of too much
glucose or too little buffering in the medium, lower levels of toxin
antigens are produced, and more rapid degradation of the toxin occurs.

Recently Mikesell et al. have demonstrated that there is a relationship
between a large plasmid in B. anthracis and these toxin antigens
(P. Mikesell, B. E. Ivins, and J. D. Ristroph, Fed. Proc., 41:1390,
1982). The recovery of this plasmid was enhanced by using R medium.
This medium has proved to be a significant tool in several other
laboratories which are critically dependent upon the facile production
of biologically and serologically functional toxin antigens. The
capability to produce increased quantities of anthrax toxin antigens in
the defined medium described in this study will further aid investigators
in their study of mechanisms of action of the toxin components and in their
attempts to develop a more efficacious human vaccine.

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TABLE 1. Components of 1095, casamino acids, and R media

	mg/liter		
	1095	CA[a]	R
L-Tryptophan	10	52	35
Glycine	22	15	65
L-Cystine		12	25
L-Tyrosine			144
L-Lysine			230
L-Valine	120		173
L-Leucine	256		230
L-Isoleucine	128		170
L-Threonine	120		120
L-Methionine	60		73
L-Aspartic acid	128		184
Na-L-Glutamate	168		612
L-Proline	30		43
L-Histidine HCl	96		55
L-Arginine HCl	21		125
L-Phenylalanine	136		125
L-Serine	42		235
L-Alanine	9		
Guanine HCl	9		
Adenosine	1.0		
Biotin	0.4		
Pyridoxal HCl	1.0		
Thiamine HCl	4.0	0.5	1.0
Glucose	1000	2000	2500

$CaCl_2 \cdot 2H_2O$	15	7.4	7.4
$MgSO_4 \cdot 7\ H_2O$	10	9.9	9.9
$MnSO_4 \cdot H_2O$	4	0.9	0.9
K_2HPO_4	870	872	3000
KH_2PO_4	680	680	
$NaHCO_3$	2500	8000	6000
$FeSO_4$	1.5		
Uracil		1.4	1.4
Adenine sulfate		2.1	2.1
Casamino acids		3600	
Activated charcoal powder		33	

[a]Additional amino acids were not added, except where noted, in excess of these found in casamino acids.

TABLE 2. Protease activity[a] in the 18-h and 30-h R medium culture supernatants of B. anthracis strains Sterne, V770-NP1-R and Vollum 1B

	CPM/0.1 ml assay sample	
Strain	18 h	30 h
Sterne	196	132
V770-NP1-R	68	205
Vollum 1B	50	33

[a]Trypsin controls, 0.1 ug/ml = 486 CPM, 0.01 ug/ml = 150 CPM, 0.001 ug/ml = 72 CPM, 0.0001 ug/ml = 24 CPM.

TABLE 3. Peak production of PA, LF, and lethal toxin in 1095, CA and R media by B. anthracis strains Sterne, V770-NP1-R, and Vollum 1B

| Strain | Reciprocal Ouchterlony titer[a] | | | | | | TU/ml medium[b] | | |
| | 1095 | | CA | | R | | | | |
	PA	LF	PA	LF	PA	LF	1095	CA	R
Sterne	1	–	4	1	4	2	5	58	84
V770-NP1-R	1	–	1	–	4	1	23	25	79
Vollum 1B	1	–	8	2	8	2	15	143	202

[a] Serologic activity was assayed by double-diffusion Ouchterlony technique using antiserum possessing both PA and LF activity.

[b] Lethal toxin activity was measured by the rat lethality assay.

FIGURE LEGENDS

FIG. 1. Growth kinetics in the R medium for B. anthracis strains Sterne (▲), V770-NP1-R (■), and Vollum 1B (●).

FIG. 2. Supernatant glucose concentration in R medium of B. anthracis strains Sterne (▲), V770-NP1-R (■), and Vollum 1B (●).

FIG. 3. Change in pH in R medium cultures of B. anthracis strains Sterne (▲), V770-NP1-R (■), and Vollum 1B (●).

FIG. 4. Supernatant protein concentration in R medium of Sterne (▲), V770-NP1-R (■), and Vollum 1B (●).

FIG. 5. Supernatant lethality producing activity in R medium of Sterne (▲), V770-NP1-R (■), and Vollum 1B (●).
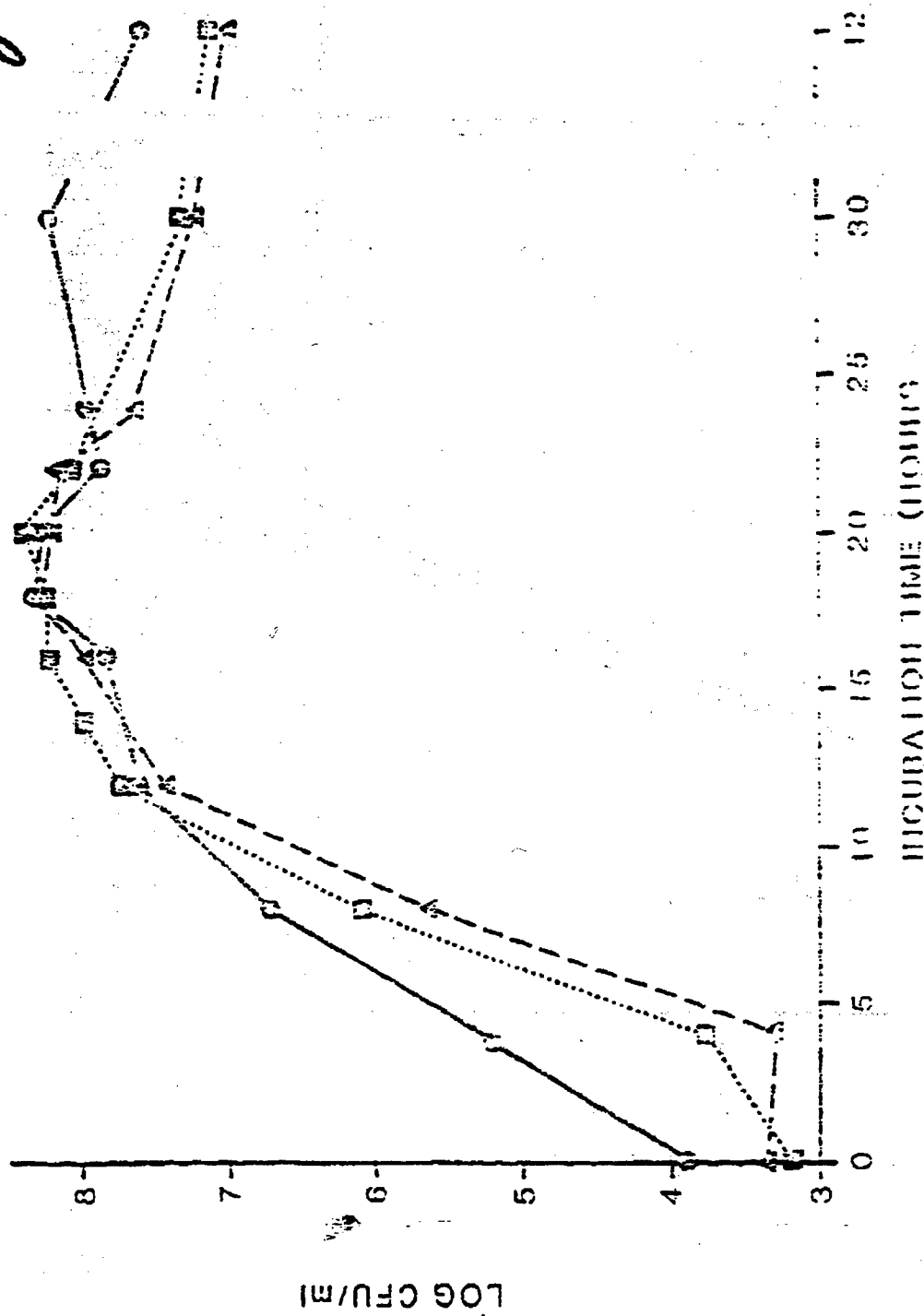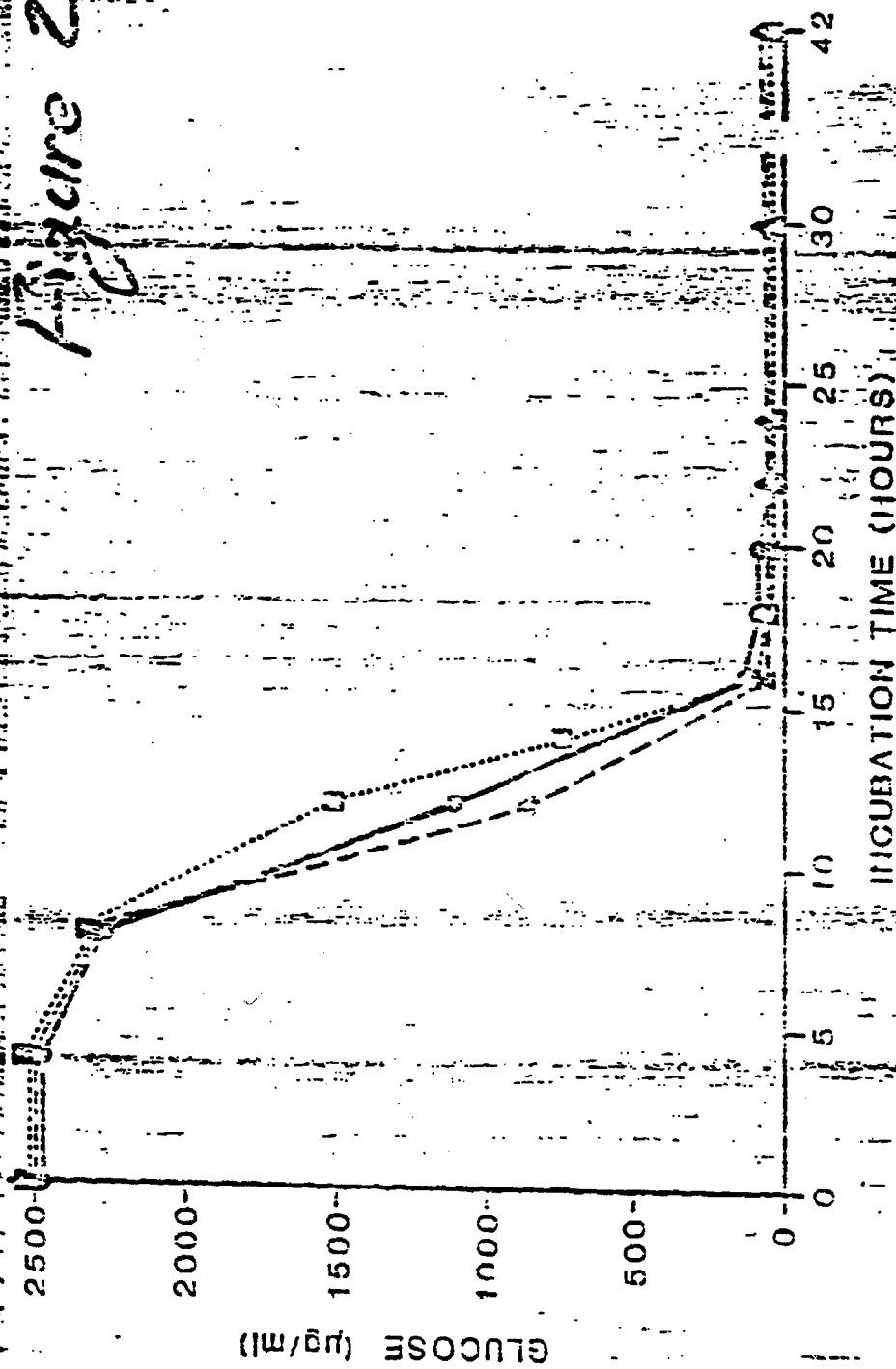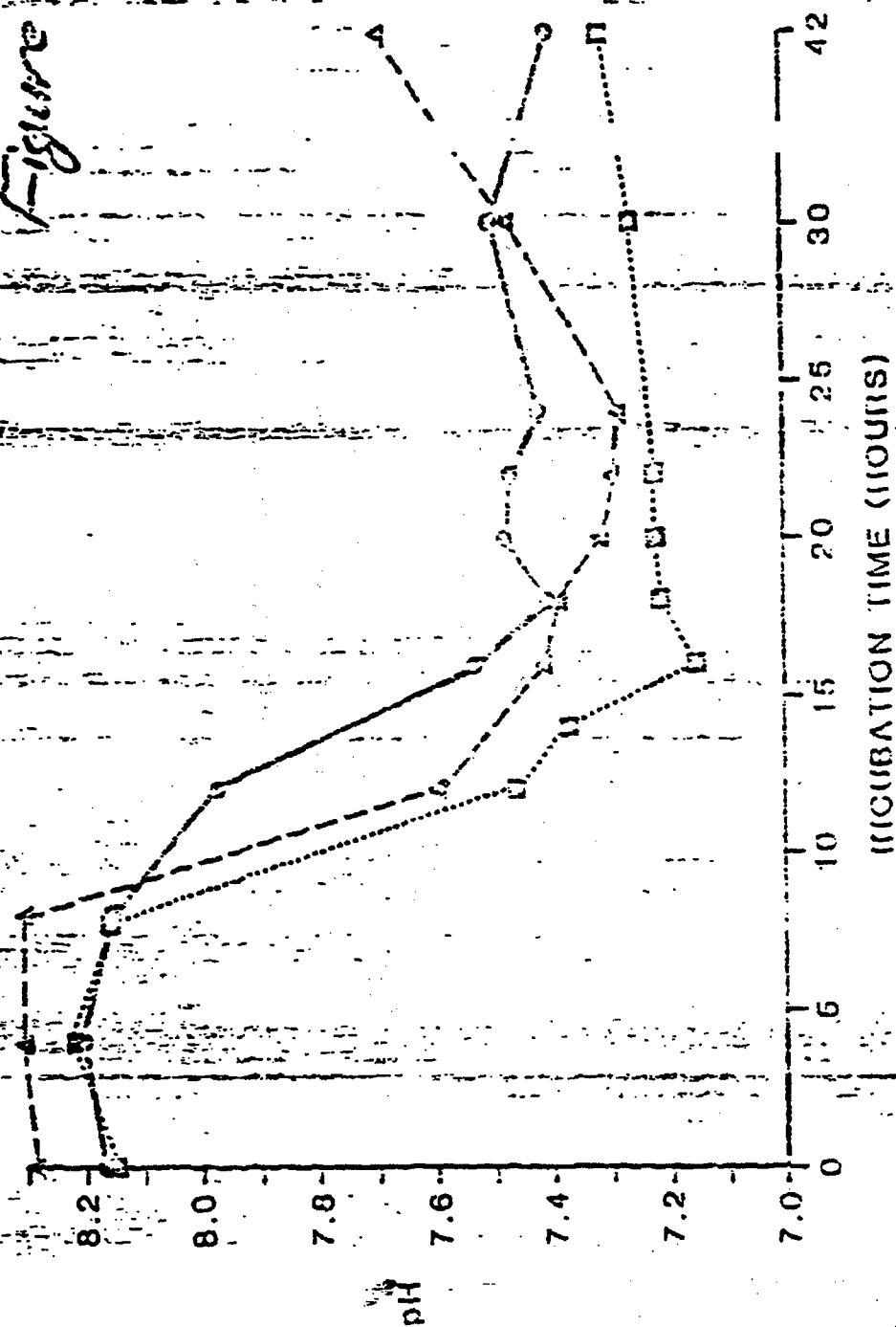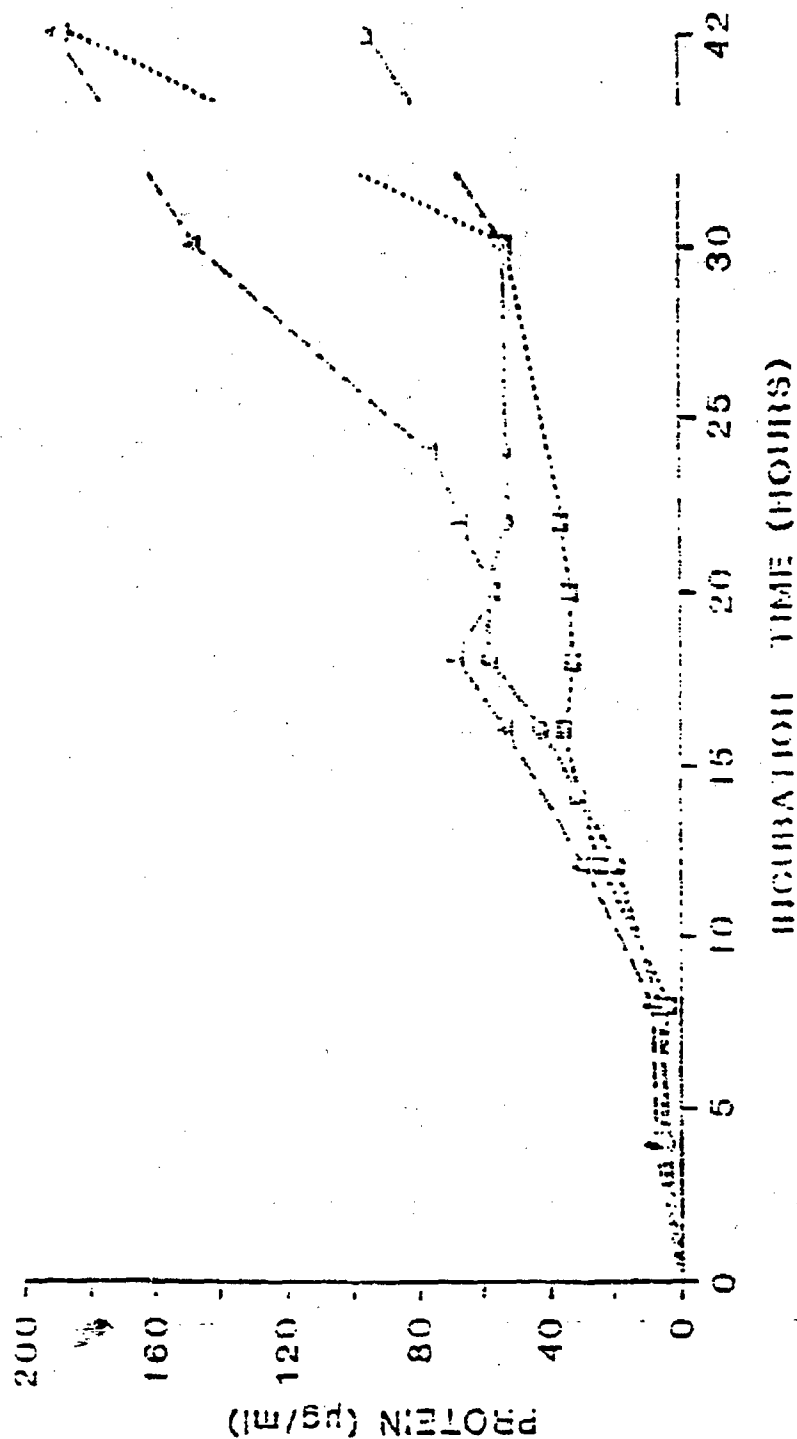
Figure 1

Figure 2

INCUBATION TIME (HOURS)

GLUCOSE (µg/ml)

Figure 3

Figure

PROTEIN (µg/ml)

INCUBATION TIME (HOURS)

Figure 5